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(71) Applicants: INTERNATIONAL GENETIC ENGINEERING, INC. [US/US]; 1545 17th Street, Santa Monica, CA 90404 (US). ONCOGEN [US/US]; 3005 First Avenue, Seattle, WA 98121 (US).

(72) Inventors: ROBINSON, Randy, R. ; 5606 W. 79th Street, Los Angeles, CA 90045 (US). LIU, Alvin, Y. ; 807 5th Street, Apt. 6, Santa Monica, CA 90403 (US). HELLSTROM, Karl, Erik ; HELLSTROM, Ingegerd ; 3925 N.E. Surber Drive, Seattle, WA 98105 (US). LEDBETTER, Jeffrey, A. ; 306 N.W. 113th Place, Seattle, WA 98177 (US).

(74) Agents: GOLDSTEIN, Jorge, A. et al.; Saidman, Sterne, Kessler & Goldstein, 1225 Connecticut Avenue N.W., Suite 300, Washington, DC 20036 (US).

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(54) Title: CHIMERIC ANTIBODY WITH SPECIFICITY TO HUMAN B CELL SURFACE ANTIGEN

(57) Abstract

A chimeric antibody with human constant region and murine variable region, having specificity to a 35 kDA polypeptide (Bp35(CD20)) expressed on the surface of human B cells, methods of production, and uses.

TITLE OF THE INVENTION

CHIMERIC ANTIBODY WITH SPECIFICITY TO HUMAN B CELL SURFACE ANTIGEN

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to recombinant DNA methods of preparing an antibody with specificity for an antigen on the surface of human B cells, genetic sequences coding therefor, as well as methods of obtaining such sequences.

Background Art

The application of cell-to-cell fusion for the production of monoclonal antibodies by Kohler and Milstein (Nature (London), 256: 495, 1975) spawned a revolution in biology equal in impact to that from the

invention of recombinant DNA cloning. Monoclonal antibodies produced from hybridomas are already widely used in clinical and basic scientific studies. Applications of human monoclonal antibodies produced by human hybridomas hold great promise for the treatment of cancer, viral and microbial infections, certain immunodeficiencies with diminished antibody production, and other diseases and disorders of the immune system.

Unfortunately, a number of obstacles exist with respect to the development of human monoclonal antibodies. This is especially true when attempting to develop therapeutically useful monoclonal antibodies which define human cell surface antigens. Many of these human cell surface antigens are not recognized as foreign antigens by the human immune system; therefore, these antigens are not immunogenic in man. By contrast, human cellular antigens which are immunogenic in mice can be used for the production of mouse monoclonal antibodies that specifically recognize the human antigens. Although such antibodies may be used therapeutically in man, repeated injections of "foreign" antibodies, such as a mouse antibody, in humans, can lead to harmful hypersensitivity reactions as well as increased rate of clearance of the circulating antibody molecules so that the antibodies do not reach their target site. Furthermore, mouse monoclonal antibodies may lack the ability to efficiently interact with human effector cells as assessed by functional assays such as antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytolysis (CDC).

Another problem faced by immunologists is that most human monoclonal antibodies obtained in cell culture are of the IgM type. When it is desirable to obtain human monoclonals of the IgG type, however, it has been necessary to use such techniques as cell sorting to identify and isolate the few cells which are producing antibodies of the IgG or other type from the majority producing antibodies of the IgM type. A need therefore exists for an efficient method of switching antibody classes, for any given antibody of a predetermined or desired antigenic specificity.

The present invention bridges both the hybridoma and genetic engineering technologies to provide a quick and efficient method, as well as products derived therefrom, for the production of a chimeric human/non-human antibody.

The chimeric antibodies of the present invention embody a combination of the advantageous characteristics of monoclonal antibodies derived from mouse-mouse hybridomas and of human monoclonal antibodies. The chimeric monoclonal antibodies, like mouse monoclonal antibodies, can recognize and bind to a human target antigen; however, unlike mouse monoclonal antibodies, the species-specific properties of the chimeric antibodies will avoid the induction of harmful hypersensitivity reactions and may allow for resistance to clearance when used in humans in vivo. Also, the inclusion of appropriate human immunoglobulin sequences can result in an antibody which efficiently interacts with human effector cells in vivo to cause tumor cell lysis and the like. Moreover, using the methods disclosed in the present invention, any desired antibody isotype can be conferred upon a particular antigen combining site.

INFORMATION DISCLOSURE STATEMENT*

Approaches to the problem of producing chimeric antibodies have been published by various authors.

Morrison, S. L. et al., Proc. Natl. Acad. Sci., USA, 81: 6851-6855 (November 1984), describe the production of a mouse-human antibody molecule of defined antigen binding specificity, produced by joining the variable region genes of a mouse antibody-producing myeloma cell line with known antigen binding specificity to human immunoglobulin constant region genes using recombinant DNA techniques. Chimeric genes were constructed, wherein the heavy chain variable region exon from the myeloma cell line S107 were joined to human IgG1 or IgG2 heavy chain constant region exons, and the light chain variable region exon from the same myeloma to the human kappa light chain exon. These genes were transfected into mouse myeloma cell lines. Transformed cells producing chimeric mouse-human antiphosphocholine antibodies were thus developed.

Morrison, S. L. et al., European Patent Publication No. 173494 (published March 5, 1986), disclose chimeric "receptors" (e.g. antibodies) having variable regions derived from one species and constant regions derived from another. Mention is made of utilizing cDNA cloning to construct the genes, although no details of cDNA cloning or priming are shown. (see pp 5, 7 and 8).

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* Note: The present Information Disclosure Statement is subject to the provisions of 37 C.F.R. 1.97(b). In addition, Applicants reserve the right to demonstrate that their invention was made prior to any one or more of the mentioned publications.

Boulianne, G. L. et al., Nature, 312: 643 (December 13, 1984), also produced antibodies consisting of mouse variable regions joined to human constant regions. They constructed immunoglobulin genes in which the DNA segments encoding mouse variable regions specific for the hapten trinitrophenyl (TNP) were joined to segments encoding human mu and kappa constant regions. These chimeric genes were expressed as functional TNP binding chimeric IgM.

For a commentary on the work of Boulianne et al. and Morrison et al., see Munro, Nature, 312: 597 (December 13, 1984), Dickson, Genetic Engineering News, 5, No. 3 (March 1985), or Marx, Science, 229: 455 (August 1985).

Neuberger, M. S. et al., Nature, 314: 268 (March 25, 1985), also constructed a chimeric heavy chain immunoglobulin gene in which a DNA segment encoding a mouse variable region specific for the hapten 4-hydroxy-3-nitrophenacetyl (NP) was joined to a segment encoding the human epsilon region. When this chimeric gene was transfected into the J558L cell line, an antibody was produced which bound to the NP hapten and had human IgE properties.

Neuberger, M.S. et al., have also published work showing the preparation of cell lines that secrete hapten-specific antibodies in which the Fc portion has been replaced either with an active enzyme moiety (Williams, G. and Neuberger, M.S. Gene 43:319, 1986) or with a polypeptide displaying c-myc antigenic determinants (Nature, 312:604, 1984).

Neuberger, M. et al., PCT Publication WO 86/01533, (published March 13, 1986) also disclose production of

chimeric antibodies (see p. 5) and suggests, among the technique's many uses the concept of "class switching" (see p. 6).

Taniguchi, M., in European Patent Publication No. 171 496 (published February 19, 1985) discloses the production of chimeric antibodies having variable regions with tumor specificty derived from experimental animals, and constant regions derived from human. The corresponding heavy and light chain genes are produced in the genomic form, and expressed in mammalian cells.

Takeda, S. et al., Nature, 314: 452 (April 4, 1985) have described a potential method for the construction of chimeric immunoglobulin genes which have intron sequences removed by the use of a retrovirus vector. However, an unexpected splice donor site caused the deletion of the V region leader sequence. Thus, this approach did not yield complete chimeric antibody molecules.

Cabilly, S. et al., Proc. Natl. Acad. Sci., USA, 81: 3273-3277 (June 1984), describe plasmids that direct the synthesis in E. coli of heavy chains and/or light chains of anti-carcinoembryonic antigen (CEA) antibody. Another plasmid was constructed for expression of a truncated form of heavy chain (Fd') fragment in E. coli. Functional CEA-binding activity was obtained by in vitro reconstitution, in E. coli extracts, of a portion of the heavy chain with light chain.

Cabilly, S., et al., European Patent Publication 125023 (published November 14, 1984) describes chimeric immunoglobulin genes and their presumptive products

as well as other modified forms. On pages 21, 28 and 33 it discusses cDNA cloning and priming.

Boss, M. A., European Patent Application 120694 (published October 3, 1984) shows expression in E. coli of non-chimeric immunoglobulin chains with 4-nitrophenyl specificity. There is a broad description of chimeric antibodies but no details (see p. 9).

Wood, C. R. et al., Nature, 314: 446 (April, 1985) describe plasmids that direct the synthesis of mouse anti-NP antibody proteins in yeast. Heavy chain mu antibody proteins appeared to be glycosylated in the yeast cells. When both heavy and light chains were synthesized in the same cell, some of the protein was assembled into functional antibody molecules, as detected by anti-NP binding activity in soluble protein prepared from yeast cells.

Alexander, A. et al., Proc. Nat. Acad. Sci. USA, 79: 3260-3264 (1982), describe the preparation of a cDNA sequence coding for an abnormally short human Ig gamma heavy chain (OMM gamma^3 HCD serum protein) containing a 19- amino acid leader followed by the first 15 residues of the V region. An extensive internal deletion removes the remainder of the V and the entire C_H1 domain. This is cDNA coding for an internally deleted molecule.

Dolby, T. W. et al., Proc. Natl. Acad. Sci., USA, 77: 6027-6031 (1980), describe the preparation of a cDNA sequence and recombinant plasmids containing the same coding for mu and kappa human immunoglobulin polypeptides. One of the recombinant DNA molecules contained codons for part of the CH_3 constant region domain and the entire 3' noncoding sequence.

Seno, M. et al., Nucleic Acids Research, 11: 719-726 (1983), describe the preparation of a cDNA sequence and recombinant plasmids containing the same coding for part of the variable region and all of the constant region of the human IgE heavy chain (epsilon chain).

Kurokawa, T. et al., ibid, 11: 3077-3085 (1983), show the construction, using cDNA, of three expression plasmids coding for the constant portion of the human IgE heavy chain.

Liu, F. T. et al., Proc. Nat. Acad. Sci., USA, 81: 5369-5373 (September 1984), describe the preparation of a cDNA sequence and recombinant plasmids containing the same encoding about two-thirds of the CH_2, and all of the C_H3 and C_H4 domains of human IgE heavy chain.

Tsujimoto, Y. et al., Nucleic Acids Res., 12: 8407-8414 (November 1984), describe the preparation of a human V lambda cDNA sequence from an Ig lambda-producing human Burkitt lymphoma cell line, by taking advantage of a cloned constant region gene as a primer for cDNA synthesis.

Murphy, J., PCT Publication WO 83/03971 (published November 24, 1983) discloses hybrid proteins made of fragments comprising a toxin and a cell-specific ligand (which is suggested as possibly being an antibody).

Tan, et al., J. Immunol. 135:8564 (November, 1985), obtained expression of a chimeric human-mouse immunoglobulin genomic gene after transfection into mouse myeloma cells.

Jones, P. T., et al., Nature 321.552 (May 1986) constructed and expressed a genomic construct where

CDR domains of variable regions from a mouse monolonal antibody were used to substitute for the corresponding domains in a human antibody.

Sun, L.K., et al., Hybridoma 5 suppl. 1 S17 (1986), describes a chimeric human/mouse antibody with potential tumor specificty. The chimeric heavy and light chain genes are genomic constructs and expressed in mammalian cells.

Sahagan et al., J. Immun. 137:1066-1074 (August 1986) describe a chimeric antibody with specificity to a human tumor associated antigen, the genes for which are assembled from genomic sequences.

For a recent review of the field see also Morrison, S.L., Science 229: 1202-1207 (September 20, 1985) and Oi, V. T., et al., BioTechniques 4:214 (1986).

The Oi, et al., paper is relevant as it argues that the production of chimeric antibodies from cDNA constructs in yeast and/or bacteria is not necessarily advantageous.

See also Commentary on page 835 in Biotechnology 4 (1986).

SUMMARY OF THE INVENTION

The invention provides a genetically engineered chimeric antibody of desired variable region specificity and constant region properties, through gene cloning and expression of light and heavy chains. The cloned immunoglobulin gene products can be produced by expression in genetically engineered cells.

The application of oligodeoxyribonucleotide synthesis, recombinant DNA cloning, and production of specific immunoglobulin chains in various prokaryotic

and eukaryotic cells provides a means for the large scale production of a chimeric human/mouse monoclonal antibody with specificity to a human B cell surface antigen.

The invention provides cDNA sequences coding for immunoglobulin chains comprising a constant human region and a variable, non-human, region. The immunoglobulin chains can be either heavy or light.

The invention provides gene sequences coding for immunoglobulin chains comprising a cDNA variable region of the desired specificity. These can be combined with genomic constant regions of human origin.

The invention provides sequences as above, present in recombinant DNA molecules in vehicles such as plasmid vectors, capable of expression in desired prokaryotic or eukaryotic hosts.

The invention provides hosts capable of producing, by culture, the chimeric antibodies and methods of using these hosts.

The invention also provides individual chimeric immunoglobulin chains, as well as complete assembled molecules having human constant regions and variable regions with a human B cell surface antigen specificity, wherein both variable regions have the same binding specificity.

Among other immunoglobulin chains and/or molecules provided by the invention are:

(a) a complete functional, immunoglobulin molecule comprising:

(i) two identical chimeric heavy chains comprising a variable region with a human B cell surface antigen specificity and human constant region and

(ii) two identical all (i.e. non-chimeric) human light chains.

(b) a complete, functional, immunoglobulin molecule comprising:

(i) two identical chimeric heavy chains comprising a variable region as indicated, and a human constant region, and

(ii) two identical all (i.e. non-chimeric) non-human light chains.

(c) a monovalent antibody, i.e., a complete, functional immunoglobulin molecule comprising:

(i) two identical chimeric heavy chains comprising a variable region as indicated, and a human constant region, and

(ii) two different light chains, only one of which has the same specificity as the variable region of the heavy chains. The resulting antibody molecule binds only to one end thereof and is therefore incapable of divalent binding.

Genetic sequences, especially cDNA sequences, coding for the aforementioned combinations of chimeric chains or of non-chimeric chains are also provided herein.

The invention also provides for a genetic sequence, especially a cDNA sequence, coding for the variable region of desired specificity of an antibody molecule heavy and/or light chain, operably linked to a sequence coding for a polypeptide different than an immunoglobulin chain (e.g., an enzyme). These sequences can be assembled by the methods of the invention, and expressed to yield mixed-function molecules.

The use of cDNA sequences is particularly advantageous over genomic sequences (which contain introns), in that cDNA sequences can be expressed in bacteria or other hosts which lack appropriate RNA splicing systems.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 shows the DNA rearrangements and the expression of immunoglobulin mu and gamma heavy chain genes. This is a schematic representation of the human heavy chain gene complex (not shown to scale). Heavy chain variable V region formation occurs through the proper joining of V_H, D and J_H gene segments. This generates an active mu gene. A different kind of DNA rearrangement called "class switching" relocates the joined V_H, D and J_H region from the vicinity of mu constant C region to that of another heavy chain C region (switching to gamma is diagrammed here).

FIGURE 2 shows the known nucleotide sequences of human and mouse J regions. Consensus sequences for the J regions are shown below the actual sequences. The oligonucleotide sequence below the mouse kappa J region consensus sequence is a Universal Immunoglobulin Gene (UIG) oligonucleotide. Note that there are only a few J regions with relatively conserved sequences, especially near the constant regions, in each immunoglobulin gene locus.

FIGURE 3 shows the nucleotide sequences of the mouse J regions. Shown below are the oligonucleotide primers UIG-H and UIG-K. Note that each contains a restriction enzyme site. They can be used as primers for the synthesis of cDNA complementary to the vari-

able region of mRNA, and can also be used to mutagenize, in vitro, cloned cDNA.

FIGURE 4 Human Constant Domain Modules. The human C gamma 1 clone, pGMH6, was isolated from the cell line GM2146. The sequence at its J_H-C_H1 junction is shown. Two restriction enzyme sites are useful as joints in recombining the C_H1 gene with different V_H genes. The ApaI site is 16 nucleotide residues into the C_H1 coding domain of Human gamma 1; and is used in a previous construction of a mouse-human chimeric heavy-chain immunoglobulin. The BstEII site is in the J_H region, and is used in the construction described in this application.

The human C_K clone, pGML60, is a composite of two cDNA clones, one isolated from GM1500 (pK2-3), the other from GM2146 (pGML1). The J_K-C_K junction sequence shown comes from pK2-3. In vitro mutagenesis using the oligonucleotide, J_KHindIII, was carried out to engineer a HindIII site 14 nucleotide residues 5' of the J-C junction. This changes a human GTG codon into a CTT codon.

FIGURE 5 shows the nucleotide sequence of the V region of the 2H7 V_H cDNA clone pH2-11. The sequence was determined by the dideoxytermination method using M13 subclones of gene fragments. Open circles denote amino acid residues confirmed by peptide sequence. A sequence homologous to $D_{SP.2}$ in the CDR3 region is underlined. The NcoI site at 5' end was converted to a SalI site by using SalI linkers.

FIGURE 6 shows the nucleotide sequence of the V region of the 2H7 V_K cDNA clone pL2-12. The oligonucleotide primer used for site-directed mutagenesis is shown below the J_K5 segment. Open circles denote amino acid residues confirmed by peptide sequence.

FIGURE 7 shows the construction of the light and heavy chain expression plasmids pING2106 (panel a) and pING2101 (panel B). The SalI to BamHI fragment from pING2100 is identical to the SalI to BamHI fragment from pING2012E (see panel C). A linear representation of the circular plasmid pING2012E is shown in panel C. The 6.6 Kb SalI to BamHI fragment contains sequences from pSV2-neo, pUC12, M8alphaRX12, and pL1. The HindIII site in pSV2-neo was destroyed before assembly of pING2012E by HindIII cleavage, fill-in, and religation.

FIGURE 8 shows the structure of several chimeric 2H7-V_H expression plasmids. pING2107 is a gpt version of the light chain plasmid, pING2106. The larger ones are two-gene plasmids. pHL2-11 and pHL2-26 contain both H and L genes, while pLL2-25 contains two L genes. They were constructed by joining an NdeI fragment containing either an H or L gene to partially digested (with NdeI) pING2106.

FIGURE 9 shows a summary of the sequence alterations made in the construction of the 2H7 chimeric antibody expression plasmids. Residues underlined in the 5' untranslated region are derived from the cloned mouse kappa and heavy-chain genes. Residues circled in the V/C boundary result from mutagenesis operations to engineer restriction enzyme sites in this region.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

INTRODUCTION

Generally, antibodies are composed of two light and two heavy chain molecules. Light and heavy chains are divided into domains of structural and functional homology. The variable domains of both the light (V_L) and the heavy (V_H) chains determine recognition and specificity. The constant region domains of light (C_L) and heavy (C_H) chains confer important biological

GENETIC PROCESSES AND PRODUCTS

The invention provides a novel approach cloning and production of a human/mouse chime: body with specificity to a human B cell surfa gen. The antigen is a polypeptide or com polypeptide bound by the 2H7 monoclonal antil cribed in Clark et al. Proc. Natl. Acad. Sci 82:1766-1770 (1985). This antigen is a phospl designated (Bp35(CD20)) and is only expressed of the B cell lineage. Murine monoclonal a: to this antigen have been made before described in Clark et al., supra; see also S· P., et al., J. Immunol. 125:1678-1685 (1980).

The method of production combines five ele

(1) Isolation of messenger RNA (mRNA) mouse hybridoma line producing the mc antibody, cloning and cDNA p: therefrom;

(2) Preparation of Universal Immunoglobu (UIG) oligonucleotides, useful as and/or probes for cloning of the region gene segments in the light a chain mRNA from the hybridoma cell l cDNA production therefrom;

(3) Preparation of constant region gene modules by cDNA preparation and clo genomic gene preparation and cloning;

(4) Construction of complete heavy or lig coding sequences by linkage of th:

in the human sequence or at another desired location near a boundary of the constant region. An alternative method utilizes genomic C region clones as the source for C region module vectors.

Third, cloned V region segments generated as above are excised and ligated to light or heavy chain C region module vectors. For example, one can clone the complete human kappa light chain C region and the complete human $gamma_1$ C region. In addition, one can modify the human $gamma_1$ region to introduce a termination codon and thereby obtain a gene sequence which encodes the heavy chain portion of an F_{ab} molecule.

The coding sequences having operationally linked V and C regions are then transferred into appropriate expression vehicles for expression in appropriate hosts, prokaryotic or eukaryotic. Operationally linked means in-frame joining of coding sequences to derive a continuously translatable gene sequence without alterations or interruptions of the triplet reading frame.

One particular advantage of using cDNA genetic sequences in the present invention is the fact that they code continuously for immunoglobulin chains, either heavy or light. By "continuously" is meant that the sequences do not contain introns (i.e. are not genomic sequences, but rather, since derived from mRNA by reverse transcription, are sequences of contiguous exons). This characteristic of the cDNA sequences provided by the invention allows them to be expressible in prokaryotic hosts, such as bacteria, or in lower eukaryotic hosts, such as yeast.

Another advantage of using cDNA cloning method is the ease and simplicity of obtaining variable region gene modules.

The terms "constant" and "variable" are used functionally to denote those regions of the immunoglobulin chain, either heavy or light chain, which code for properties and features possessed by the variable and constant regions in natural non-chimeric antibodies. As noted, it is not necessary for the complete coding region for variable or constant regions to be present, as long as a functionally operating region is present and available.

Expression vehicles include plasmids or other vectors. Preferred among these are vehicles carrying a functionally complete human constant heavy or light chain sequence having appropriate restriction sites engineered so that any variable heavy or light chain sequence with appropriate cohesive ends can be easily inserted thereinto. Human constant heavy or light chain sequence-containing vehicles are thus an important embodiment of the invention. These vehicles can be used as intermediates for the expression of any desired complete heavy or light chain in any appropriate host.

One preferred host is yeast. Yeast provides substantial advantages for the production of immunoglobulin light and heavy chains. Yeasts carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies now exist which utilize strong promoter sequences and high copy number plasmids which can be used for overt production of the desired proteins in yeast. Yeast re-

cognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e. prepeptides) (Hitzman, et al., 11th International Conference on Yeast, Genetics and Molecular Biology, Montpelier, France, September 13-17, 1982).

Yeast gene expression systems can be routinely evaluated for the level of heavy and light chain production, protein stability, and secretion. Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeasts are grown in mediums rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcription control signals. For example, the promoter and terminator signals of the iso-1-cytochrome C (CYC-1) gene can be utilized.

The following approach can be taken to develop and evaluate optimal expression plasmids for the expression of cloned immunoglobulin cDNAs in yeast.

(1) The cloned immunoglobulin DNA linking V and C regions is attached to different transcription promoters and terminator DNA fragments;

(2) The chimeric genes are placed on yeast plasmids (see, for example, Broach, J.R. in Methods in Enzymology - Vol. 101:307 ed. Wu, R. et al., 1983));

(3) Additional genetic units such as a yeast leader peptide may be included on immunoglobulin DNA constructs to obtain antibody secretion.

(4) A portion of the sequence, frequently the first 6 to 20 codons of the gene sequence may be modified to represent preferred yeast codon usage.

(5) The chimeric genes are placed on plasmids used for integration into yeast chromosomes.

The following approaches can be taken to simultaneously express both light and heavy chain genes in yeast.

(1) The light and heavy chain genes are each attached to a yeast promoter and a terminator sequence and placed on the same plasmid. This plasmid can be designed for either autonomous replication in yeast or integration at specific sites in the yeast chromosome.

(2) The light and heavy chain genes are each attached to a yeast promoter and terminator sequence on separate plasmids containing different selectable markers. For example, the light chain gene can be placed on a plasmid containing the trp1 gene as a selectable marker, while the heavy chain gene can be placed on a plasmid containing ura3 as a selectable marker. The plasmids can be designed for either autonomous replication in yeast or integration at specific sites in yeast chromosomes. A yeast strain defective for both selectable markers is either simultaneously or sequentially transformed with the plasmid containing the light chain gene and with the plasmid containing the heavy chain gene.

(3) The light and heavy chain genes are each attached to a yeast promoter and terminator sequence on separate plasmids each containing different selectable markers as described in (2) above. A yeast mating type "a" strain defective in the selectable markers found on the light and heavy chain expression plasmids (trp1 and ura3 in the above example) is transformed with the plasmid containing the light chain gene by selection for one of the two selectable markers (trp1 in the above example). A yeast mating type "alpha" strain defective in the same selectable markers as the "a" strain (i.e. trp1 and ura3 as examples) is transformed with a plasmid containing the heavy chain gene by selection for the alternate selectable marker (i.e. ura3 in the above example). The "a" strain containing the light chain plasmid (phenotype: Trp^+ Ura^- in the above example) and the strain containing the heavy chain plasmid (phenotype: Trp^- Ura^+ in the above example) are mated and diploids are selected which are prototrophic for both of the above selectable markers (Trp^+ Ura^+ in the above example).

Among bacterial hosts which may be utilized as transformation hosts, E. coli K12 strain 294 (ATCC 31446) is particularly useful. Other microbial strains which may be used include E. coli X1776 (ATCC 31537). The aforementioned strains, as well as E. coli W3110 (ATCC 27325) and other enterobacteria such as Salmonella typhimurium or Serratia marcescens, and various Pseudomonas species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. For example, E. coli is readily transformed using pBR322, a plasmid derived from an E. coli species (Bolivar, et al., Gene, 2: 95 (1977)). pBR322 contains genes for ampicillin and tetracycline resistance, and thus provides easy means for identifying transformed cells. The pBR322 plasmid or other microbial plasmids must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the beta-lactamase (penicillinase) and lactose (beta-galactosidase) promoter systems (Chang et al., Nature, 275: 615 (1978); Itakura et al., Science, 198:1056 (1977)); and tryptophan promoter systems (Goeddel et al., Nucleic Acids Research, 8: 4057 (1980); EPO Publication No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized.

For example, a genetic construct for any heavy or light chimeric immunoglobulin chain can be placed under the control of the leftward promoter of bacteriophage lambda (P_L). This promoter is one of the strongest known promoters which can be controlled. Control is exerted by the lambda repressor, and adjacent restriction sites are known.

The expression of the immunoglobulin chain sequence can also be placed under control of other regulatory sequences which may be "homologous" to the organism in its untransformed state. For example, lactose dependent E. coli chromosomal DNA comprises a lactose or lac operon which mediates lactose digestion by elaborating the enzyme beta-galactosidase. The lac control elements may be obtained from bacteriophage lambda pLAC5, which is infective for E. coli. The lac promoter-operator system can be induced by IPTG.

Other promoter/operator systems or portions thereof can be employed as well. For example, arabinose, colicine E1, galactose, alkaline phosphatase, tryptophan, xylose, tac, and the like can be used.

Other preferred hosts are mammalian cells, grown in vitro in tissue culture, or in vivo in animals. Mammalian cells provide post-translational modifications to immunoglobulin protein molecules including leader peptide removal, correct folding and assembly of heavy and light chains, proper glycosylation at correct sites, and secretion of functional antibody protein.

Mammalian cells which may be useful as hosts for the production of antibody proteins include cells of lymphoid origin, such as the hybridoma Sp2/0-Ag14 (ATCC CRL 1581) or the myleoma P3X63Ag8 (ATCC TIB 9), and its derivatives. Others include cells of fibroblast origin, such as Vero (ATCC CRL 81) or CHO- K1 (ATCC CRL 61).

Several possible vector systems are available for the expression of cloned heavy chain and light chain genes in mammalian cells. One class of vectors re-

lies upon the integration of the desired gene sequences into the host cell genome. Cells which have stably integrated DNA can be selected by simultaneously introducing drug resistance genes such as E. coli gpt (Mulligan, R. C. and Berg, P., Proc. Natl. Acad. Sci., USA, 78: 2072 (1981)) or Tn5 neo (Southern, P. J. and Berg, P., J. Mol. Appl. Genet., 1: 327 (1982)). The selectable marker gene can be either linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection (Wigler, M. et al., Cell, 16: 77 (1979)). A second class of vectors utilizes DNA elements which confer autonomously replicating capabilities to an extrachromosomal plasmid. These vectors can be derived from animal viruses, such as bovine papillomavirus (Sarver, N. et al., Proc. Natl. Acad. Sci., USA, 79: 7147 (1982)), polyoma virus (Deans, R. J. et al., Proc. Natl. Acad. Sci., USA, 81: 1292 (1984)), or SV40 virus (Lusky, M. and Botchan, M., Nature, 293: 79 (1981)).

Since an immunoglobulin cDNA is comprised only of sequences representing the mature mRNA encoding an antibody protein additional gene expression elements regulating transcription of the gene and processing of the RNA are required for the synthesis of immunoglobulin mRNA. These elements may include splice signals, transcription promoters, including inducible promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, H. and Berg, P., Mol. Cell Biol., 3: 280 (1983); Cepko, C. L. et al., Cell, 37: 1053 (1984); and Kaufman, R. J., Proc. Natl. Acad. Sci., USA, 82: 689 (1985).

An additional advantage of mammalian cells as hosts is their ability to express chimeric immunoglobulin genes which are derived from genomic sequences. Thus, mammalian cells may express chimeric immunoglobulin genes which are comprised of a variable region cDNA module plus a constant region which is composed in whole or in part of genomic sequences. Several human constant region genomic clones have been described (Ellison, J. W. et al., Nucl. Acids Res., 10: 4071 (1982), or Max, E. et al., Cell, 29: 691 (1982)). The use of such genomic sequences may be convenient for the simultaneous introduction of immunoglobulin enhancers, splice signals, and transcription termination signals along with the constant region gene segment.

Different approaches can be followed to obtain complete H_2L_2 antibodies.

First, one can separately express the light and heavy chains followed by in vitro assembly of purified light and heavy chains into complete H_2L_2 IgG antibodies. The assembly pathways used for generation of complete H_2L_2 IgG molecules in cells have been extensively studied (see, for example, Scharff, M., Harvey Lectures, 69: 125 (1974)). In vitro reaction parameters for the formation of IgG antibodies from reduced isolated light and heavy chains have been defined by Beychok, S., Cells of Immunoglobulin Synthesis, Academic Press, New York, page 69, 1979.

Second, it is possible to co-express light and heavy chains in the same cells to achieve intracellular association and linkage of heavy and light chains into complete H_2L_2 IgG antibodies. The co-expression

can occur by using either the same or different plasmids in the same host.

POLYPEPTIDE PRODUCTS

The invention provides "chimeric" immunoglobulin chains, either heavy or light. A chimeric chain contains a constant region substantially similar to that present in a natural human immunoglobulin, and a variable region having the desired antigenic specificity of the invention, i.e., to the specified human B cell surface antigen.

The invention also provides immunoglobulin molecules having heavy and light chains associated so that the overall molecule exhibits any desired binding and recognition properties. Various types of immunoglobulin molecules are provided: monovalent, divalent, molecules with chimeric heavy chains and non-chimeric light chains, or molecules with the invention's variable binding domains attached to moieties carrying desired functions.

Antibodies having chimeric heavy chains of the same or different variable region binding specificity and non-chimeric (i.e., all human or all non-human) light chains, can be prepared by appropriate association of the needed polypeptide chains. These chains are individually prepared by the modular assembly methods of the invention.

USES

The antibodies of the invention having human constant region can be utilized for passive immunization, especially in humans, without negative immune reac-

tions such as serum sickness or anaphylactic shock. The antibodies can, of course, also be utilized in prior art immunodiagnostic assays and kits in detectably labelled form (e.g., enzymes, ^{125}I, ^{14}C, fluorescent labels, etc.), or in immunobilized form (on polymeric tubes, beads, etc.), in labelled form for in vivo imaging, wherein the label can be a radioactive emitter, or an NMR contrasting agent such as a carbon-13 nucleus, or an X-ray contrasting agent, such as a heavy metal nucleus. The antibodies can also be used for in vitro localization of the antigen by appropriate labelling.

The antibodies can be used for therapeutic purposes, by themselves, in complement mediated lysis, or coupled to toxins or therapeutic moieties, such as ricin, etc.

Mixed antibody-enzyme molecules can be used for immunodiagnostic methods, such as ELISA. Mixed antibody-peptide effector conjugates can be used for targeted delivery of the effector moiety with a high degree of efficacy and specificity.

Specifically, the chimeric antibodies of this invention can be used for any and all uses in which the murine 2H7 monoclonal antibody can be used, with the obvious advantage that the chimeric ones are more compatible with the human body.

Having now generally described the invention, the same will be further understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

EXPERIMENTAL

Materials and Methods

Tissue Culture Cell Lines

The human cell lines GM2146 and GM1500 were obtained from the Human Mutant Cell Repository (Camden, New Jersey) and cultured in RPMI1640 plus 10% fetal bovine serum (M. A. Bioproducts). The cell line Sp2/0 was obtained from the American Type Culture Collection and grown in Dulbecco's Modified Eagle Medium (DMEM) plus 4.5 g/l glucose (M. A. Bioproducts) plus 10% fetal bovine serum (Hyclone, Sterile Systems, Logan, Utah). Media were supplemented with penicillin/streptomycin (Irvine Scientific, Irvine, California).

Recombinant Plasmid and Bacteriophage DNAs

The plasmids pBR322, pL1 and pUC12 were purchased from Pharmacia P-L Biochemicals (Milwaukee, Wisconsin). The plasmids pSV2-neo and pSV2-gpt were obtained from BRL (Gaithersburg, Maryland), and are available from the American Type Culture Collection (Rockville, Maryland). pHu-gamma-1 is a subclone of the 8.3 Kb HindIII to BamHI fragment of the human IgG1 chromosomal gene. An isolation method for of the human IgG1 chromosomal gene is described by Ellison, J. W. et al., Nucl. Acids Res., 10: 4071 (1982). M8alphaRX12 contains the 0.7 Kb XbaI to EcoRI fragment containing the mouse heavy chain enhancer from the J-C intron region of the M603 chromosomal gene (Davis, M. et al., Nature, 283:733, 1979) inserted into M13mp10. DNA manipulations involving purification of plasmid DNA by buoyant density centrifugation, restriction endonuclease digestion, purification of DNA fragments

by agarose gel electrophoresis, ligation and transformation of E. coli were as described by Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, (1982) or other procedures. Restriction endonucleases and other DNA/RNA modifying enzymes were purchased from Boehringer-Mannheim (Indianapolis, Indiana), BRL, New England Biolabs (Beverly, Massachusetts) and Pharmacia P-L.

Oligonucleotide Preparation

Oligonucleotides were either synthesized by the triester method of Ito et al. (Nucl. Acids Res., 10: 1755 (1982)), or were purchased from ELESEN, Los Angeles, California. Tritylated, deblocked oligonucleotides were purified on Sephadex-G50, followed by reverse-phase HPLC with a 0-25% gradient of acetonitrile in 10mM triethylamine-acetic acid, pH 7.2, on a C18 Bondapak column (Waters Associates). Detritylation was in 80% acetic acid for 30 min., followed by evaporation thrice. Oligonucleotides were labeled with [gamma-^{32}P]ATP by T4 polynucleotide kinase.

RNA Preparation and Analysis

Total cellular RNA was prepared from tissue culture cells by the method of Auffray, C. and Rougeon, F. (Eur. J. Biochem., 107: 303 (1980)) or Chirgwin, J. M. et al. (Biochemistry, 18: 5294 (1979)). Preparation of poly(A)$^+$ RNA, methyl-mercury agarose gel electrophoresis, and "Northern" transfer to nitrocellulose were as described by Maniatis, T. et al., supra. Total cellular RNA or poly(A)$^+$ RNA was directly bound to nitrocellulose by first treating the RNA with formaldehyde (White, B. A. and Bancroft, F. C., J. Biol. Chem., 257: 8569 (1982)). Hybridization to filterbound

RNA was with nick-translated DNA fragments using conditions described by Margulies, D. H. et al. (Nature, 295: 168 (1982)) or with ^{32}P-labelled oligonucleotide using 4xSSC, 10X Denhardt's, 100 ug/ml salmon sperm DNA at 37°C overnight, followed by washing in 4xSSC at 37°C.

cDNA Preparation and Cloning

Oligo-dT primed cDNA libraries were prepared from poly(A)$^+$ RNA from GM1500 and GM2146 cells by the methods of Land, H. et al. (Nucl. Acids Res., 9: 2251 (1981)) and Gubler, V. and Hoffman, B. J., Gene, 25: 263 (1983), respectively. The cDNA libraries were screened by hybridization (Maniatis, T., supra) with ^{32}P-labelled oligonucleotides using the procedure of de Lange et al. (Cell, 34: 891 (1983)), or with nick-translated DNA fragments.

Oligonucleotide Primer Extension and Cloning

Poly(A)$^+$ RNA (20 ug) was mixed with 1.2 ug primer in 40 ul of 64mM KCl. After denaturation at 90°C for 5 min. and then chilling in ice, 3 units Human Placental Ribonuclease Inhibitor (BRL) was added in 3 ul of 1M Tris-HCl, pH 8.3. The oligonucleotide was annealed to the RNA at 42°C for 15 minutes, then 12 ul of .05M DTT, .05M $MgCl_2$, and 1 mM each of dATP, dTTP, dCTP, and dGTP was added. 2 ul of alpha-^{32}P-dATP (400 Ci/mmol, New England Nuclear) was added, followed by 3 ul of AMV reverse transcriptase (19 units/ul, Life Sciences).

After incubation at 42°C for 105 min., 2 ul 0.5 M EDTA and 50 ul 10mM Tris, 1mM EDTA, pH 7.6 were added. Unincorporated nucleotides were removed by Sephadex G-50 spin column chromatography, and the RNA-DNA hy-

brid was extracted with phenol, then with chloroform, and precipitated with ethanol. Second strand synthesis, homopolymer tailing with dGTP or dCTP, and insertion into homopolymer tailed vectors was as described by Gubler and Hoffman, supra.

Site-Directed Mutagenesis

Single stranded M13 subclone DNA (1 ug) was combined with 20 ng oligonucleotide primer in 12.5 ul of Hin buffer (7 mM Tris-HCl, pH 7.6, 7 mM $MgCl_2$, 50 mM NaCl). After heating to 95°C in a sealed tube, the primer was annealed to the template by slowly cooling from 70°C to 37°C for 90 minutes. 2 ul dNTPs (1 mM each), 1 ul ^{32}P-dATP (10 uCi), 1 ul DTT (0.1 M) and 0.4 ul Klenow DNA PolI (2u, Boehringer Mannheim) were added and chains extended at 37°C for 30 minutes. To this was added 1 ul (10 ng) M13 reverse primer (New England Biolabs), and the heating/annealing and chain extension steps were repeated. The reaction was stopped with 2 ul of 0.5M EDTA, pH 8, plus 80 ul of 10 mM Tris-HCl, pH 7.6, 1 mM EDTA. The products were phenol extracted and purified by Sephadex G-50 spun column chromatography and ethanol precipitated prior to restriction enzyme digestion and ligation to the appropriate vector.

Transfection of Myeloma Tissue Culture Cells

The electroporation method of Potter, H. et al. (Proc. Natl. Acad. Sci., USA, 81: 7161 (1984)) was used. After transfection, cells were allowed to recover in complete DMEM for 48-72 hours, then were seeded at 10,000 to 50,000 cells per well in 96-well culture plates in the presence of selective medium. G418 (GIBCO) selection was at 0.8 mg/ml, and myco-

phenolic acid (Calbiochem) was at 6 ug/ml plus 0.25 mg/ml xanthine.

Assays for Immunoglobulin Synthesis and Secretion

Secreted immunoglobulin was measured directly from tissue culture cell supernatants. Cytoplasmic protein extract was prepared by vortexing 10^6 cells in 160 ul of 1% NP40, 0.15 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.6 and leaving the lysate at $0^{\circ}C$, 15 minutes, followed by centrifugation at 10,000 x g to remove insoluble debris.

A double antibody sandwich ELISA (Voller, A. et al., in Manual of Clinical Immunology, 2nd Ed., Eds. Rose, N. and Friedman, H., pp. 359-371, 1980) using affinity purified antisera was used to detect specific immunoglobulins. For detection of human IgG, the plate-bound antiserum is goat anti-human IgG (KPL, Gaithersburg, Maryland) at 1/1000 dilution, while the peroxidase-bound antiserum is goat anti-human IgG (KPL or Tago, Burlingame) at 1/4000 dilution. For detection of human immunoglobulin kappa, the plate-bound antiserum is goat anti-human kappa (Tago) at 1/500 dilution, while the peroxidase-bound antiserum is goat anti-human kappa (Cappel) at 1/1000 dilution.

EXAMPLE 1

A Chimeric Mouse-Human Immunoglobulin with Specificity for a Human B Cell Surface Antigen

(1) Antibody 2H7.

The 2H7 mouse monoclonal antibody (gamma 2b, kappa) recognizes a human B-cell surface antigen, (Bp35(CD20)) Clark, E.A., et al., Proc. Natl. Acad. Sci., U.S.A. 82:1766 (1985)). The (Bp35(CD20))

molecules presumably play a role in B-cell activation. The antibody 2H7 does not react with stem cells which are progenitors of B cells epithelial, mesenchymal and fibroblastic cells of other organs.

(2) Identification of J Sequences in the Immunoglobulin mRNA of 2H7.

Frozen cells were thawed on ice for 10 minutes and then at room temperature. The suspension was diluted with 15 ml PBS and the cells were centrifuged down. They were resuspended, after washes in PBS, in 16 ml 3M LiCl, 6M urea and disrupted in a polytron shear. The preparation of mRNA and the selection of the poly(A+) fraction were carried out according to Auffray, C. and Rougeon, F., Eur. J. Biochem. 107:303, 1980.

The poly (A+) RNA from 2H7 was hybridized individually with labeled J_H1, J_H2, J_H3 and J_H4 oligonucleotides under conditions described by Nobrega et al. Anal. Biochem 131:141, 1983). The products were then subjected to electrophoresis in a 1.7% agarose-TBE gel. The gel was fixed in 10% TCA, blotted dry and exposed for autoradiography. The result showed that the 2H7 V_H contains J_H1, J_H2, orJ_H4 but not J_H3 sequences.

For the analysis of the V_K mRNA, the dot-blot method of White and Bancroft J. Biol. Chem. 257:8569, (1982) was used. Poly (A+) RNA was immobilized on nitrocellulose filters and was hybridized to labeled probe-oligonucleotides at 40° in 4xSSC. These experiments show that 2H7 contains J_K5 sequences.

(3) V Region cDNA Clones.

A library primed by oligo (dT) on 2H7 poly (A+) RNA was screened for kappa clones with a mouse C_K

region probe. From the 2H7 library, several clones were isolated. A second screen with a 5' J_K5 specific probe identified the 2H7 (J_K5) light-chain clones. Heavy chain clones of 2H7 were generated by priming the poly(A+) RNA with the UIGH(BstEII) oligonucleotide (see Figure 3), and identified by screening with the UIGH(BstEII) oligonucleotide.

The heavy and light chain genes or gene fragments from the V_H and V_K cDNA clones pH2-11 and pL2-12 were inserted into M13 bacteriophage vectors for nucleotide sequence analysis. The complete nucleotide sequences of the variable region of these clones were determined (FIGURES 5 and 6) by the dideoxy chain termination method. These sequences predict V region amino acid compositions that agree well with the observed compositions, and predict peptide sequences which have been verified by direct amino acid sequencing of portions of the V regions.

The nucleotide sequences of the cDNA clones show that they are immunoglobulin V region clones as they contain amino acid residues diagnostic of V domains (Kabat et al., Sequences of Proteins of Immunological Interest; U.S. Dept of HHS, 1983).

The 2H7 V_H has the J_H1 sequence. The 2H7 V_L is from the V_K-KpnI family (Nishi et al. Proc. Nat. Acd. Sci. USA 82:6399, 1985), and uses J_K5. The cloned 2H7 V_L predicts an amino acid sequence which was confirmed by amino acid sequencing of peptides from the 2H7 light chain corresponding to residues 81-100. The cloned 2H7 V_H predicts an amino acid sequence confirmed also by peptide sequencing, namely residues 1-12.

(4) In Vitro Mutagenesis to Engineer Restriction Enzyme Sites in the J Region for Joining to a Human C-Module, and to Remove Oligo (dC) Sequences 5' to the V Modules.

For the 2H7 V_K, the J-region mutagenesis primer J_KHindIII, as shown in FIGURE 6, was utilized. A human C_K module derived from a cDNA clone was also mutagenized to contain the HindIII sequence (see Figure 4). The mutagenesis reaction was performed on M13 subclones of these genes. The frequency of mutant clones ranged from 0.5 to 1% of the plaques obtained.

It had been previously observed that the oligo (dC) sequence upstream of the AUG codon in a V_H chimeric gene interferes with proper splicing in one particular gene construct. It was estimated that perhaps as much as 70% of the RNA transcripts had undergone the mis-splicing, wherein a cryptic 3' splice acceptor in the leader sequence was used. Therefore the oligo (dC) sequence upstream of the initiator AUG was removed in all of the clones.

In one approach, an oligonucleotide was used which contains a SalI restriction site to mutagenize the 2H7 V_K clone. The primer used for this oligonucleotide-directed mutagenesis is a 22-mer which introduces a SalI site between the oligo (dC) and the initiator met codon (FIGURE 6).

In a different approach, a convenient NcoI site was utilized to delete the 5' untranslated region and oligo (dC) of the 2H7 V_H clone (see Figure 5).

The human C gamma 1 gene module is a cDNA derived from GM2146 cells (Human Genetic Mutant Cell Repository, Newark, New Jersey). This C gamma 1 gene module

was previously combined with a mouse V_H gene module to form the chimeric expression plasmid pING2012E (Figure 7C).

(5) Chimeric 2H7 Expression Plasmids.

A 2H7 chimeric heavy chain expression plasmid was derived from the replacement of the V_H module of pING2012E with the V_H cDNA modules to give the expression plasmid pING2101 (FIGURE 7B). This plasmid directs the synthesis of chimeric 2H7 heavy chain when transfected into mammalian cells.

For the 2H7 light chain chimeric gene, the SalI to HindIII fragment of the mouse V_K module was joined to the human C_K module by the procedure outlined in FIGURE 7A, forming pING2106. Replacement of the neo sequence with the E. coli gpt gene derived from pSV2-gpt resulted in pING2107, which expresses 2H7 chimeric light chain and confers mycophenolic acid resistance when transfected into mammalian cells.

The inclusion of both heavy and light chain chimeric genes in the same plasmid allows for the introduction into transfected cells of a 1:1 gene ratio of heavy and light chain genes leading to a balanced gene dosage. This may improve expression and decrease manipulations of transfected cells for optimal chimeric antibody expression. For this purpose, the DNA fragments derived from the chimeric heavy and light chain genes of pING2101 and pING2106 were combined into the expression plasmids pHL2-11 and pHL2-26 (FIGURE 8). The pHL2-11 and pHL2-26 plasmids each contain a selectable neo^R marker and separate transcription units for each chimeric gene, each gene including a mouse heavy chain enhancer.

The modifications and V-C joint regions of the 2H7 chimeric genes are summarized in FIGURE 9.

(6) Stable Transfection of Mouse Lymphoid Cells for the Production of Chimeric Antibody.

Electroporation was used (Potter et al. supra; Toneguzzo et al. Mol. Cell Biol. 6:703 1986) for the introduction of 2H7 chimeric expression plasmid DNA into mouse Sp2/0 cells. The electroporation technique gave a transfection frequency of 10^{-4} x 10^{-5} for the Sp2/0 cells.

The expression plasmids, pING2101 and pING2106, were digested with NdeI and the DNA was introduced into Sp2/0 cells by electroporation. Transformant 1D6 was obtained which secretes chimeric 2H7 antibody. Antibody isolated from this cell line was used for the functional assays done to characterize the chimeric antibody. We have also obtained transformants from experiments using the two-gene plasmids.

(7) Purification of Chimeric 2H7 Antibody Secreted in Tissue Culture.

a. 1D6 (Sp2/0.pING2101/pING2106.1D6) cells were grown in culture medium [DMEM (Gibco #320-1965), supplemented with 10% Fetal Bovine Serum (Hyclone #A-1111-D), 10mM HEPES, 1x Glutamine-Pen-Strep (Irvine Scientific #9316) to 1 x 10^6 cell/ml.

b. The cells were then centrifuged at 400xg and resuspended in serum-free culture medium at 2 x 10^6 cell/ml for 18-24 hr.

c. The medium was centrifuged at 4000 RPM in a JS-4.2 rotor (3000xg) for 15 min.

d. 1.6 liter of supernatant was then filtered through a 0.45 micron filter and then concentrated over a YM30 (Amicon Corp.) filter to 25ml.

e. The conductance of the concentrated supernatant was adjusted to 5.7-5.6 mS/cm CDM 80 radiometer and the pH was adjusted to 8.0.

f. The supernatant was centrifuged at 2000xg, 5 min., and then loaded onto a 40 ml DEAE column, which was preequilibrated with 10mM sodium phosphate, pH8.0.

g. The flow through fraction was collected and loaded onto a 1ml protein A-Sepharose (Sigma) column preequilibrated with 10mM sodium phosphate, pH8.0.

h. The column was washed first with 6ml 10mM sodium phosphate buffer pH 8.0, followed by 8ml 0.1M sodium citrate pH 3.5, then by 6ml 0.1M citric acid (pH 2.2). Fractions of 0.5ml were collected in tubes containing 50ul 2M Tris base (Sigma).

i. The bulk of the IgG was in the pH 3.5 elution and was pooled and concentrated over Centricon 30 (Amicon Corp.) to approximately .06ml.

j. The buffer was changed to PBS (10mM sodium phosphate pH 7.4, 0.15M NaCl) in Centricon 30 by repeated diluting with PBS and reconcentrating.

k. The IgG solution was then adjusted to 0.10ml and bovine serum albumin (Fraction V, U.S. Biochemicals) was added to 1.0% as a stabilizing reagent.

(9) Chimeric 2H7 Antibody, Like the Mouse 2H7 Antibody, Specifically Binds to Human B Cells.

First, the samples were tested with a binding assay, in which cells of both an 2H7 antigen-positive and an 2H7 antigen-negative cell line were incubated with standard mouse monoclonal antibody 2H7 with chimeric 2H7 antibody derived from the cell culture super-

natants, followed by a second reagent, fluorescein-isothiocyanate (FITC)-conjugated goat antibodies to human (or mouse, for the standard) immunoglobulin.

Binding Assays. Cells from a human B cell line, T51, were used. Cells from human colon carcinoma line C3347 were used as a negative control, since they, according to previous testing, do not express detectable amounts of the 2H7 antigen. The target cells were first incubated for 30 min at 4°C with either the chimeric 2H7 or with mouse 2H7 standard, which had been purified from mouse ascites. This was followed by incubation with a second, FITC-labelled, reagent, which for the chimeric antibody was goat-anti-human immunoglobulin, obtained from TAGO (Burlingame, CA), and used at a dilution of 1:50. For the mouse standard, it was goat-anti-mouse immunoglobulin, also obtained from TAGO and used at a dilution of 1:50. Antibody binding to the cell surface was determined using a Coulter Model EPIC-C cell sorter.

As shown in Table I, both the chimeric and the mouse standard 2H7 bound significantly, and to approximately the same extent, to the positive T51 line. They did not bind above background to the 2H7 negative C-3347 line.

Functional Assays.

In previous studies, antibody 2H7 was tested for antibody-dependent cellular cytotoxicity (ADCC) measured by its ability to lyse ^{51}Cr-labelled human B lymphoma cells in the presence of human peripheral blood leukocytes as the source of effector cells. It was also tested for its ability to lyse ^{51}Cr labelled human B cells in the presence of human serum as the source of complement. These tests were carried out as

previously described for mouse monoclonal anti-carcinoma antibody L6, which can mediate ADCC, as well as complement-mediated cytoxicity, CDC. The techniques used and the data described for the L6 antibody have been previously described. Hellstrom, et al., Proc. Natl. Acad Sci. U.S.A. 83: 7059-7063 (1986).

Chimeric 2H7, but not mouse 2H7 antibody, will be able to mediate both ADCC and CDC against human B lymphoma cells. Thus a hybridoma producing a non-functional mouse antibody can be converted to a hybridoma producing a chimeric antibody with ADCC and CDC activities. Such a chimeric antibody is a prime candidate for the treatment or imaging of B cell disorders, such as leukemias, lymphomas, and the like.

This invention therefore provides a method for making biologically functional antibodies when starting with a hybridoma which produces antibody which has the desired specificity for antigen but lacks biological effector functions such as ADCC and CDC.

Conclusions.

The results presented above demonstrate that the chimeric 2H7 antibody binds to (Bp35(CD20)) antigen positive human B cells to approximately the same extent as the mouse 2H7 monoclonal antibody. This is significant because the 2H7 antibody defines a surface phosphoprotein antigen (Bp35(CD20)), of about 35,000 daltons, which is expressed on the cells of B cell lineage. The 2H7 antibody does not bind detectably to various other cells such as fibroblasts, endothelial cells, or epithelial cells in the major organs or the stem cell precursors which give rise to B cells.

Although the prospect of attempting tumor therapy using monoclonal antibodies is attractive, with some partial tumor regressions being reported, to date such monoclonal antibody therapy has been met with limited success (Houghton et al., February 1985, Proc. Natl. Acad. Sci. 82:1242-1246). Murine monoclonal anti-(Bp35(CD20)) antibody has been used for therapy of B cell malignancies (Press, et al.,) Blood: Feb. 1987, in press). The therapeutic efficacy of mouse monoclonal antibodies (which are the ones that have been tried so far) appears to be too low for most practical purposes. Because of the "human" properties which may make the chimeric 2H7 monoclonal antibodies more resistant to clearance and less immunogenic in vivo, the chimeric 2H7 monoclonal antibodies will be advantageously used not only for therapy with unmodified chimeric antibodies, but also for development of various immunoconjugates with drugs, toxins, immunomodulators, isotopes, etc., as well as for diagnostic purposes such as in vivo imaging of B-cell tumors (for example, lymphomas and leukemias) using appropriately labelled chimeric 2H7 antibodies. Such immunoconjugation techniques are known to those skilled in the art and can be used to modify the chimeric 2H7 antibody molecules of the present invention. The chimeric 2H7 antibody, by virtue of its having the human constant portion, will possess biological activity in complement-dependent and antibody-dependent cytotoxicity which the mouse 2H7 does not.

An illustrative cell line secreting chimeric 2H7 antibody was deposited prior to the U.S. filing date at the ATCC, Rockville Maryland. This is a transfected hybridoma (corresponds to 1D6 cells supra) ATCC HB 9303.

The present invention is not to be limited in scope by the cell lines deposited since the deposited embodiment is intended as a single illustration of one aspect of the invention and all cell lines which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those shown in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

TABLE 1

Binding Assays Of Chimeric 2H7 Antibody and Mouse 2H7 Monoclonal Antibody to a B cell Line Expressing (Bp35(CD20)) and a Cell Line Not Expressing This Antigen.

	Binding Ratio* for T51 B Cells	
Antibody	GAM	GAH
2H7 Mouse	37	ND
2H7 Chimeric	ND	29
L6 Mouse	1	ND
	Binding Ratio* for C3347 Cells	
	GAM	GAH
2H7 Mouse	1.4	ND
2H7 Chimeric	ND	1.3
L6 Mouse	110	ND

*All assays were conducted using an antibody concentration of 10ug/ml. The binding ratio is the number of times brighter a test sample is than a control sample treated with GAM(FITC-Conjugated goat anti-mouse) or GAH (FITC conjugated goat anti-human) alone. A ratio of 1 means that the test sample is just as bright as the control; a ratio of 2 means the test sample is twice as bright as the control and so on.

ND - not done

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 39, line 11 of the description [1]

A. IDENTIFICATION OF DEPOSIT [5]

Further deposits are identified on an additional sheet ☐ [6]

Name of depositary institution [4]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit [4]	Accession Number [4]
02 January 1987	HB 9303

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C273 (mouse SP2/0 hybridoma cells carrying plasmids pING 2101 and pING 2106)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (If the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer) Annette Short

☐ The date of receipt (from the applicant) by the International Bureau [10]

WHAT IS NEW AND INTENDED TO BE COVERED BY LETTERS PATENT:

1. A polynucleotide molecule comprising a cDNA sequence coding for the variable region of an immunoglobulin chain having specificity to a 35-kDa polypeptide (Bp35(CD20)) expressed on the surface of B cells.

2. The molecule of claim 1 wherein said chain is a heavy chain.

3. The molecule of claim 1 wherein said chain is a light chain.

4. The molecule of claim 1 which further comprises an additional sequence coding for the constant region of a human immunoglobulin chain, both said sequences in operable linkage with each other.

5. The molecule of claim 4 wherein said additional sequence is a cDNA sequence.

6. The molecule of claim 4 wherein said additional sequence is a genomic sequence.

7. The molecule of claim 1 which is a recombinant DNA molecule.

8. The molecule of claim 7 which is in double-stranded DNA form.

9. The molecule of claim 7 which is an expressible vehicle.

10. The molecule of claim 9 wherein said vehicle is a plasmid.

11. A prokaryotic host transformed with the molecule of claim 4.

12. The host of claim 11 which is a bacterium.

13. A eukaryotic host transfected with the molecule of claim 4.

14. The host of claim 13 which is yeast or a mammalian cell.

15. A heavy immunoglobulin chain comprising a constant human region and a variable region having specificity to a 35 kDa polypeptide (Bp35(CD20)) expressed on the surface of human B cells.

16. A light immunoglobulin chain comprising a constant human region and a variable region having specificity to a 35 kDa polypeptide (Bp35(CD20)) expressed on the surface of human B cells.

17. A chimeric antibody molecule comprising two light chains and two heavy chains, each of said chains comprising a constant human region and a variable region having specificity to a 35 kDa polypeptide (Bp35(CD20)) expressed on the surface of human B cells.

18. The antibody of claim 17 in detectably labelled form.

19. The antibody of claim 17 immobilized on an aqueous-insoluble solid phase.

20. A process of preparing an immunoglobulin heavy chain having a constant human region and a variable region having specificity to a 35 kDa polypeptide (Bp35(CD20)) expressed on the surface of human B cells which comprises:

culturing a host capable of expressing said chain under culturing conditions and

recovering from said culture said heavy chain.

21. A process of preparing an immunoglobulin light chain having a constant human region and a variable region with specificity to a 35 kDa polypeptide (Bp35(CD20)) expressed on the surface of human B cells which comprises:

culturing a host capable of expressing said chain under culturing conditions; and

recovering from said culture said light chain.

22. A process of preparing a chimeric immunoglobulin containing a heavy chain and a light chain, each of said heavy and light chains having a constant human region and a variable region with specificity to a 35 kDa polypeptide (Bp35(CD20)) expressed on the surface of human B cells which comprises:

culturing a host capable of expressing said heavy chain, or said light chain, or both, under culturing conditions; and

recovering from said culture said chimeric immunoglobulin molecule.

23. The process of any of claims 20, 21 or 22 wherein said host is prokaryotic.

24. The process of any of claims 20, 21 or 22 wherein said host is eukaryotic.

25. An immunoassay method for the detection of a 35 kDa polypeptide normally expressed on the surface of B cells in a sample, which comprises:

contacting said sample with the antibody of claim 17 and

detecting whether said antibody binds to said antigen.

26. An in vivo or in vitro imaging method to detect an antigen comprising a 35 kDa polypeptide normally expressed on the surface of B cells which comprises contacting said antigen with the labelled antibody of claim 18 and detecting said antibody.

27. A method of killing cells carrying an antigen thereon, which antigen comprising a 35 kDa polypeptide normally expressed on the surface of B cells which comprises:

contacting said cells with the antibody of claim 17.

28. The method of claim 27 wherein said killing occurs by complement mediated lysis of said cells.

29. The method of claim 27 wherein said killing occurs by ADCC.

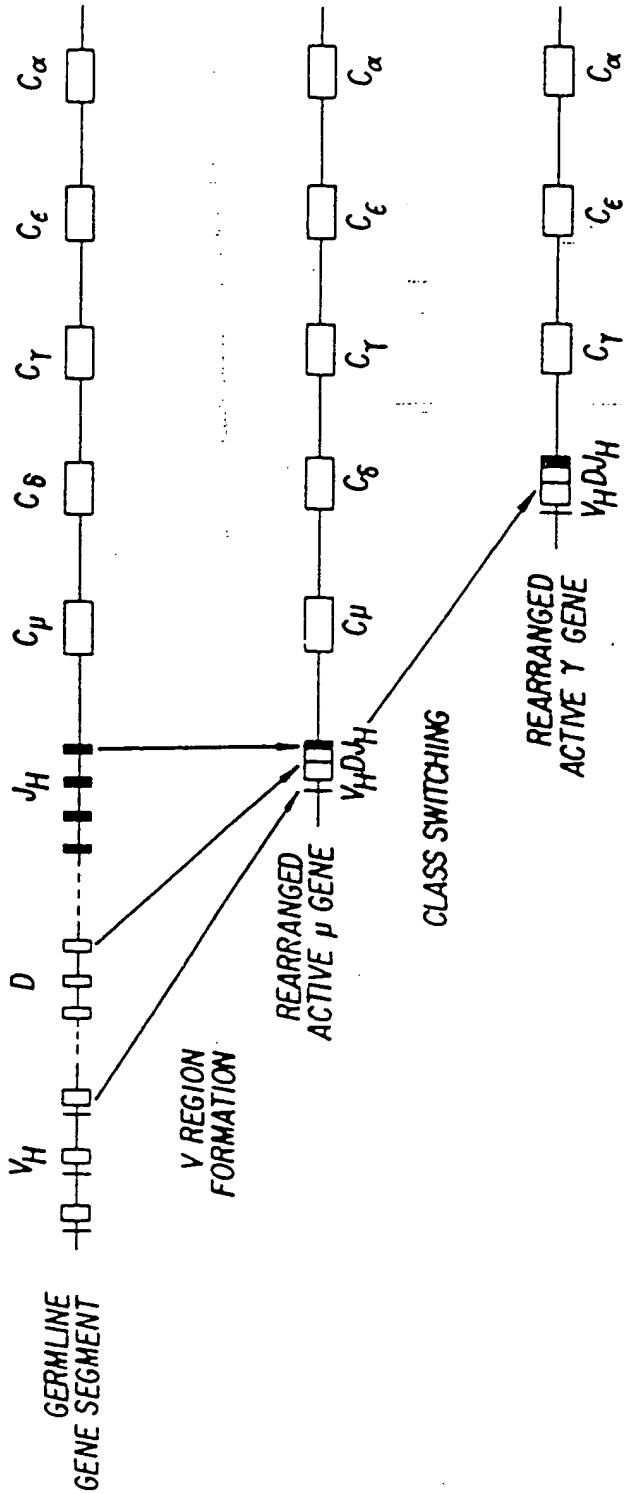

GERMLINE GENE SEGMENT
V_H
D
J_H
C_μ
C_δ
C_γ
C_ϵ
C_α
V REGION FORMATION
REARRANGED ACTIVE μ GENE
V_HDJ_H
CLASS SWITCHING
REARRANGED ACTIVE γ GENE
V_HDJ_H

FIG. 1

Ig heavy chain J-C region

human heavy chain J regions J | CH1

```
JH1    GCTGAATACTTCCAGCACTGGGGCCAGGGCACCCTGGTCACCGTCTCCTCAG
JH2  CTACTGGTACTTCGATCTCTGGGGCCGTGGCACCCTGGTCACTGTCTCCTCAG
JH3        ATGCTTTTGATGTCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCAG
JH4        ACTACTTTGACTACTGGGGCCAAGGAACCCTGGTCACCGTCTCCTCAG
JH5      ACACTGGTTCGACTCCTGGGGCCAAGGAACCCTGGTCACCGTCTCCTCAG
JH6 AT(TAC)5GGTATGGACGTCTGGGGGCAAGGGACCACGGTCACCGTCTCCTCAG
Consensus        TCGACCTCTGGGGCCAAGGAACCCTGGTCACCGTCTCCTCAG
```

mouse heavy chain J regions J | CH1

```
JH1    TACTGGTACTTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCAG
JH2          TACTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAG
JH3        CCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAG
JH4    TACTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAG
Consensus       TTTGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAG
```

Ig light chain J-C region

human Kappa J region J | C

```
JK1  GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAAC
JK2  ACACTTTTGGCCAGGGGACCAAGCTGGAGATCAAAC
JK3  TCACTTTCGGCCCTGGGACCAAAGTGGATATCAAAC
JK4  TCACTTTCGGCGGAGGGACCAAGGTGGAGATCAAAC
JK5  TCACCTTCGGCCAAGGGACACGACTGGAGATTAAAC
Consensus TTCGGCCAAGGGACCAAGGTGGAGATCAAAC
```

mouse Kappa J region J | C

```
JK1 TGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC
JK2 TACACGTTCGGAGGGGGGACCAAGCTGGAAATAAAAC
JK3 TTCACATTCAGTGATGGGACCAGACTGGAAATAAAAC
JK4 TTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAAAC
JK5 CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAAC
Consensus TTCGGTGGGGGGACCAAGCTGGAAATAAAAC
UIG[MJK]              3'TGGTTCGACCTTTATTTTG5'
```

human Lambda pseudo J region J | C

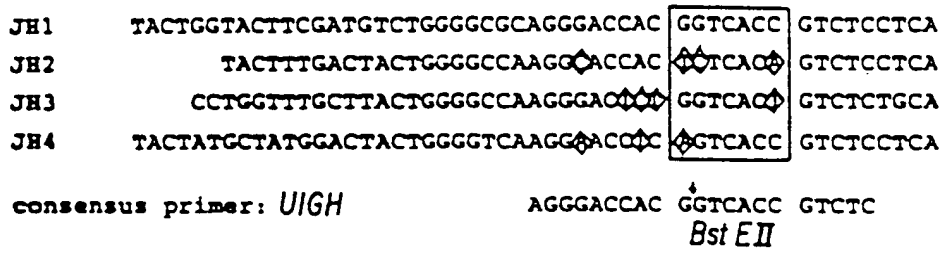
```
JPSL1 CACATGTTTGGCAGCAAGACCCAGCCCACTGTCTTAG
```

mouse Lambda J region J | C

```
JL1 TGGGTGTTCGGTGGAGGAACCAAACTGACTGTCCTAG
JL2 TATGTTTTCGGCGGTGGAACCAAGGTCACTGTCCTAG
JL3 TTTATTTTCGGCAGTGGAACCAAGGTCACTGTCCTAG
Consensus TTCGGCGGTGGAACCAAGGTCACTGTCCTAG
```

FIG.2

mouse heavy chain J segments



mouse κ light chain J segments

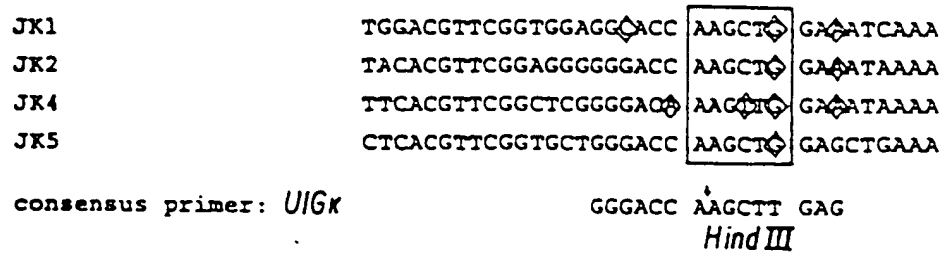


FIG. 3

pGMH6 Human $C_\gamma 1$ constant domain module

J_H

G GTC ACC GTC TCT TCA | GCC TCC ACC AAG GGC CCA TCG GTC TTC -

Bst E II

Apa I

pGML60 Human C_κ constant domain module

$J_\kappa 4$

GAT CAT CTC CCT CTC ACT TTC GGC GGA GGG ACC AAG GTG GAG ATG AAA | -

C-T

Hind III

J_κ Hind III

FIG. 4

2H7 HEAVY CHAIN VARIABLE SEQUENCE

leader

met gly phe ser arg ile phe
C_{33}GTACCTCTCTACAGTCCCTGAAGACACTGACTCTAACCATG GGA TTC AGC AGG ATC TTT

NcoI—SalI

peptide FR1

leu phe leu leu ser val thr thr gly val his ser gln ala tyr leu gln
CTC TTC CTC CTG TCA GTA ACT ACA GGT GTC CAC TCC CAG GCT TAT CTA CAG

gln ser gly ala glu leu val arg pro gly ala ser val lys met ser cys
CAG TCT GGG GCT GAG CTG GTG AGG CCT GGG GCC TCA GTG AAG ATG TCC TGC

FR1|CDR1 CDR1|FR2

lys ala ser gly tyr thr phe thr ser tyr asn met his trp val lys gln
AAG GCT TCT GGC TAC ACA TTT ACC AGT TAC AAT ATG CAC TGG GTA AAG CAG

FR2|CDR2

thr pro arg gln gly leu glu trp ile gly ala ile tyr pro gly asn gly
ACA CCT AGA CAG GGC CTG GAA TGG ATT GGA GCT ATT TAT CCA GGA AAT GGT

CDR2|FR3

asp thr ser tyr asn gln lys phe lys gly lys ala thr leu thr val asp
GAT ACT TCC TAC AAT CAG AAG TTC AAG GGC AAG GCC ACA CTG ACT GTA GAC

lys ser ser ser thr ala tyr met gln leu ser ser leu thr ser glu asp
AAA TCC TCC AGC ACA GCC TAC ATG CAG CTC AGC AGC CTG ACA TCT GAA GAC

FR3|CDR3

ser ala val tyr phe cys ala arg val val tyr tyr ser asn ser tyr trp
TCT GCG GTC TAT TTC TGT GCA AGA GTG GTG TAC TAT AGT AAC TCT TAC TGG

J_H1 DSP2

CDR3|FR4 FR4

tyr phe asp val trp gly thr gly thr thr val thr val ser
TAC TTC GAT GTC TGG GGC ACA GGG ACC ACG GTC ACC GTC TCG

BstEII J BstEII

FIG. 5

2H7 LIGHT CHAIN VARIABLE SEQUENCE

leader peptide

met asp phe gln val gln ile phe ser phe leu leu
C 23 CCCAAAATTCAAAGACAAAATG GAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA
GTC — SalI primer
| FRI
ile ser ala ser val ile ile ala arg gly gln ile val leu ser gln ser
ATC AGT GCT TCA GTC ATA ATT GCC AGA GGA CAA ATT GTT CTC TCC CAG TCT
FRI |
pro ala ile leu ser ala ser pro gly glu lys val thr met thr cys arg
CCA GCA ATC CTG TCT GCA TCT CCA GGG GAG AAG GTC ACA ATG ACT TGC AGG
CDRI CDRI |FR2
ala ser ser ser val ser tyr met his trp tyr gln gln lys pro gly ser
GGC AGC TCA AGT GTA AGT TAC ATG CAC TGG TAC CAG CAG AAG CCA GGA TCC
KpnI ↑ ↑BamHI
FR2| CDR2 CDR2|FR3
ser pro lys pro trp ile tyr ala pro ser asn leu ala ser gly val pro
TCC CCC AAA CCC TGG ATT TAT GCC CCA TCC AAC CTG GCT TCT GGA GTC CCT
ala arg phe ser gly ser gly ser gly thr ser tyr ser leu thr ile ser
GCT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC
FR3|CDR3
arg val glu ala glu asp ala ala thr tyr tyr cys gln gln trp ser phe
AGA GTG GAG GCT GAA GAT GCT GCC ACT TAT TAC TGC CAG CAG TGG AGT TTT
CDR3 | FR4 JK5 FR4
asn pro pro thr phe gly ala gly thr lys leu glu leu lys
AAC CAA CCC ACG TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA
T
JK Hind III primer

FIG. 6

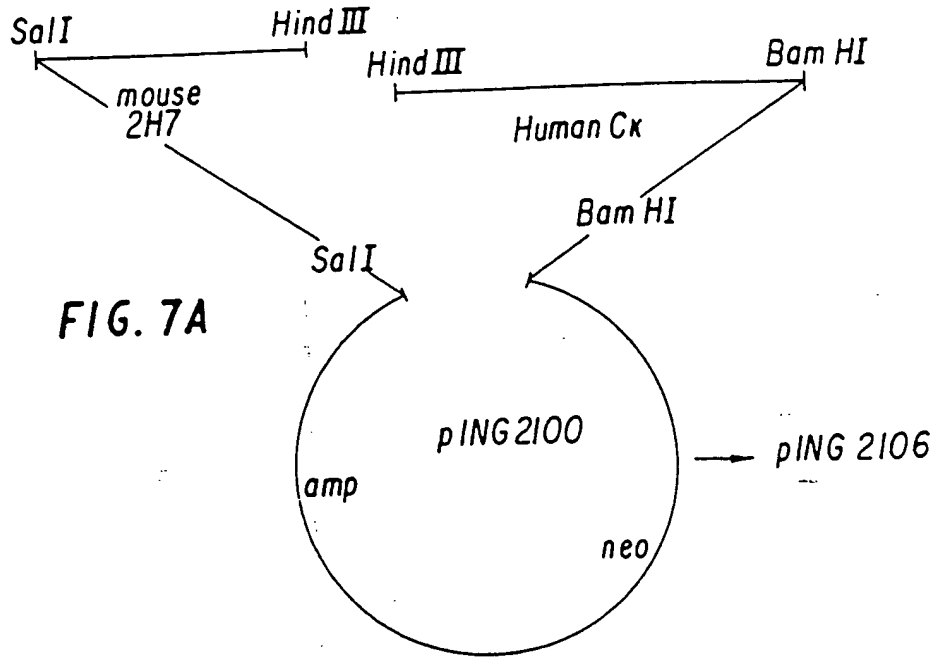


FIG. 7A

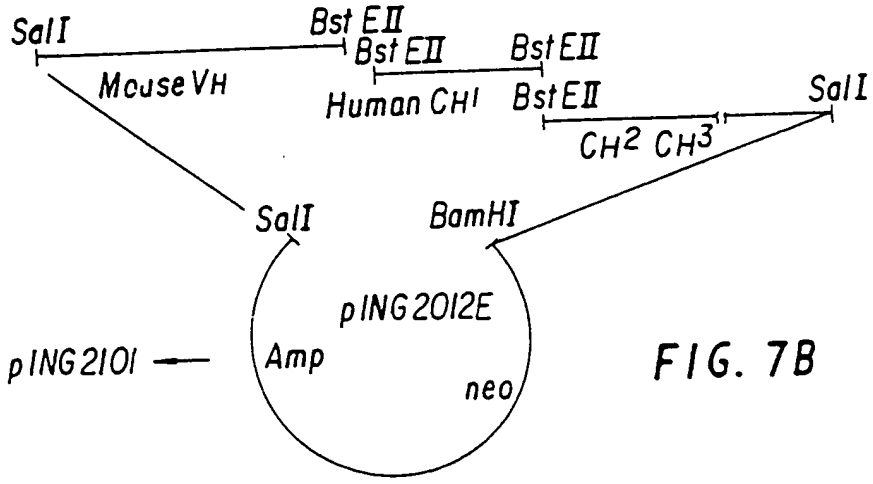


FIG. 7B

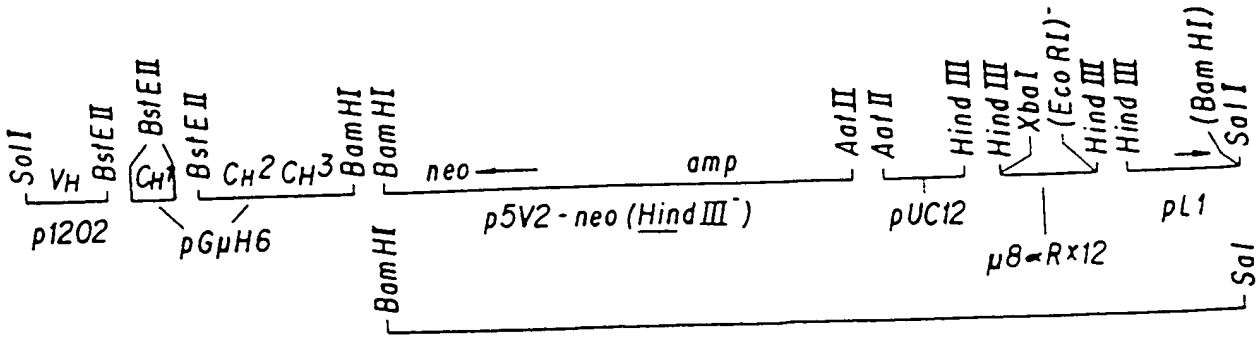


FIG. 7B

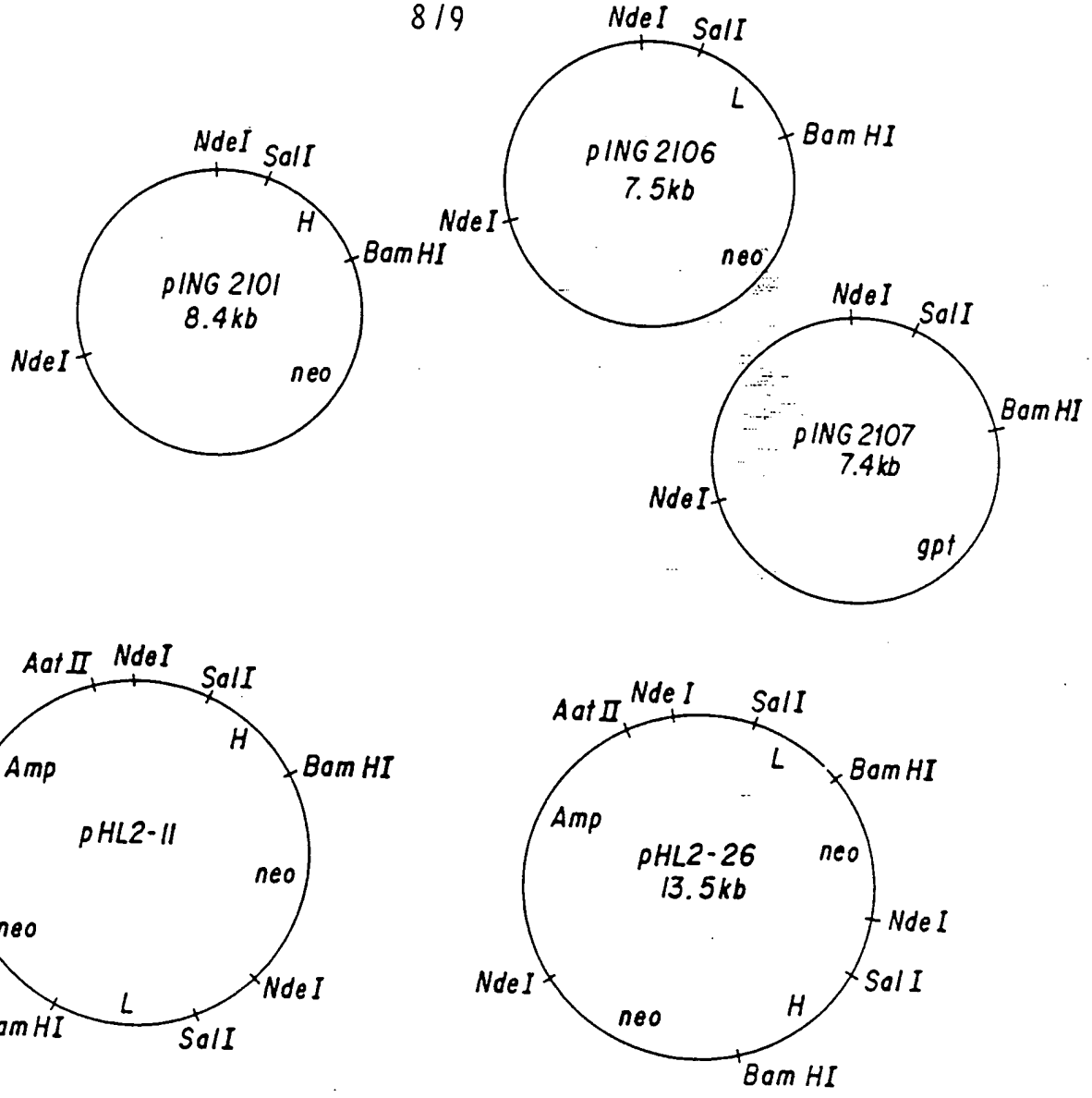
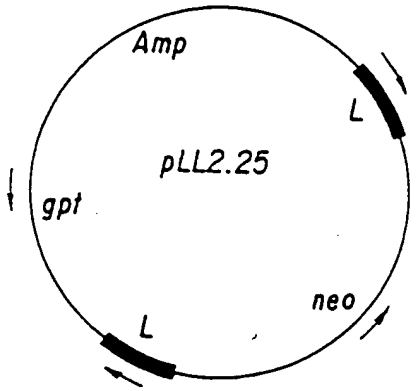

NdeI
SalI
H
BamHI
pING 2101
8.4kb
NdeI
neo
NdeI
SalI
L
BamHI
pING 2106
7.5kb
NdeI
neo
NdeI
SalI
BamHI
pING 2107
7.4kb
NdeI
gpt
AatII
NdeI
SalI
H
BamHI
Amp
pHL2-11
neo
NdeI
neo
NdeI
L
BamHI
SalI
AatII
NdeI
SalI
L
BamHI
Amp
pHL2-26
13.5kb
neo
NdeI
NdeI
SalI
neo
H
BamHI

FIG. 8


Amp
L
pLL2.25
gpt
neo
L

2H7 Chimerae

VH pH2-7(JH BstEII Clone, NcoI Cut 5'ATG → pING 2101 neo

SalI
5' GTCGA met CATGGGA

mo ho C1

joint ACGGTCACCGTCTCTTCA | GCCTCC

Vk pL2-12 (JH5) oligo(dT) clone, Jk Hind III mutagenesis. 5'SAL mutagenesis → pING 2106 neo
pING 2107 gpt

SalI
5' GTC met GAC AAA ATG GAT

mo hu Ck

joint ACC AAG CTT GAG ATG AAA | CGA ACT

FIG. 9